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(57) Abstract

A new family of tumor rejection antigen precursors, and the nucleic acid molecules which code for them, are disclosed. These tumor rejection antigen precursors are referred to as GAGE tumor rejection antigen precursors, and the nucleic acid molecules which code for them are referred to as GAGE coding molecules. Various diagnostic and therapeutic uses of the coding sequences and the tumor rejection antigens, and their precursor molecules are described. Tumor rejection antigens are also shown.

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ISOLATED, NUCLEIC ACID MOLECULES WHICH CODE FOR GAGE TUMOR REJECTION ANTIGEN, THE TUMOR REJECTION ANTIGEN, AND USES THEREOF

RELATED APPLICATION

This application is a continuation-in-part of Serial No. 08/531,662, filed September 21, 1995, which is a continuation-in-part of copending Serial No. 08/370,648 filed January 10, 1995, which is a continuation-in-part of co-pending patent application Serial No. 08/250,162 filed on May 27, 1994, which is a continuation-in-part of Serial No. 08/096,039 filed July 22, 1993. Both of these applications are incorporated by reference.

FIELD OF THE INVENTION

This invention relates to a nucleic acid molecule which codes for a tumor rejection antigen precursor. More particularly, the invention concerns genes, whose tumor rejection antigen precursor is processed, inter alia, into at least one tumor rejection antigen that is presented by HLA-Cw6 molecules. The genes in question do not appear to be related to other known tumor rejection antigen precursor coding sequences. The invention also relates to peptides presented by the HLA-Cw6 molecules, and uses thereof. Also a part of the inventions are peptides presented by HLA-A29 molecules, and uses thereof.

BACKGROUND AND PRIOR ART

The process by which the mammalian immune system recognizes and reacts to foreign or alien materials is a complex one. An important facet of the system is the T lymphocyte, or "T cell" response. This response requires that T cells recognize and interact with complexes of cell surface molecules, referred to as human leukocyte antigens ("HLAs"), or major histocompatibility complexes ("MHCs"), and peptides. The peptides are derived from larger molecules which are processed by the cells which also present the HLA/MHC molecule. See in this regard Male et al., Advanced Immunology (J.P. Lipincott Company, 1987), especially chapters 6-10.

The interaction of T cells and HLA/peptide complexes is restricted, requiring a T cell specific for a particular combination of an HLA molecule and a peptide. If a specific T cell is not present, there is no T cell response even if its partner complex is present. Similarly, there is no response if the specific complex is absent, but the T cell is present. This mechanism is involved in the immune system's response to foreign materials, in autoimmune pathologies, and in responses to cellular abnormalities. Much work has focused on the mechanisms by which proteins are processed into the HLA binding peptides. See, in this regard, Barinaga, Science 257: 880 (1992); Fremont et al., Science 257: 919 (1992); Matsumura et al., Science 257: 927 (1992); Latron et al., Science 257: 964 (1992). Also see Engelhard, Ann. Rev. Immunol. 12: 181-207 (1994).

The mechanism by which T cells recognize cellular abnormalities has also been implicated in cancer. For example, in PCT application PCT/US92/04354, filed May 22, 1992, published on November 26, 1992, and incorporated by reference, a family of genes is disclosed, which are processed into peptides which, in turn, are expressed on cell surfaces, which can lead to lysis of the tumor cells by specific CTLs cytolytic T lymphocytes, or "CTLs" hereafter. The genes are said to code for "tumor rejection antigen precursors" or "TRAP" molecules, and the peptides derived therefrom are referred to as "tumor rejection antigens" or "TRAs". See Traversari et al., Immunogenetics 35: 145 (1992); van der Bruggen et al., Science 254: 1643 (1991), for further information on this family of genes. Also, see U.S. patent application Serial Number 807,043, filed December 12, 1991, now U.S. Patent No. 5,342,774.

In U.S. patent application Serial Number 938,334, now U.S. Patent No. 5,405,940, the disclosure of which is incorporated by reference, it is explained that the MAGE-1 gene codes for a tumor rejection antigen precursor which is processed to nonapeptides which are presented by the HLA-A1 molecule. The reference teaches that given the known speci-

ficity of particular peptides for particular HLA molecules, one should expect a particular peptide to bind to one HLA molecule, but not to others. This is important, because different individuals possess different HLA phenotypes. As a result, while identification of a particular peptide as being a partner for a specific HLA molecule has diagnostic and therapeutic ramifications, these are only relevant for individuals with that particular HLA phenotype. There is a need for further work in the area, because cellular abnormalities are not restricted to one particular HLA phenotype, and targeted therapy requires some knowledge of the phenotype of the abnormal cells at issue.

In U.S. Patent Application Serial Number 008,446, filed January 22, 1993 and incorporated by reference, the fact that the MAGE-1 expression product is processed to a second TRA is disclosed. This second TRA is presented by HLA-C clone 10 molecules. The disclosure shows that a given TRAP can yield a plurality of TRAs.

U.S. Patent Application Serial Number 994,928, filed December 22, 1992, and incorporated by reference herein teaches that tyrosinase, a molecule which is produced by some normal cells (e.g., melanocytes), is processed in tumor cells to yield peptides presented by HLA-A2 molecules.

In U.S. patent application Serial No. 08/032,978, filed March 18, 1993, and incorporated by reference in its entirety, a second TRA, not derived from tyrosinase is taught to be presented by HLA-A2 molecules. The TRA is derived from a TRAP, but is coded for by a non-MAGE gene. This disclosure shows that a particular HLA molecule may present TRAs derived from different sources.

In U.S. patent application Serial No. 08/079,110, filed June 17, 1993 and incorporated by reference herein, an unrelated tumor rejection antigen precursor, the so-called "BAGE" precursor is described. The BAGE precursor is not related to the MAGE family.

The work which is presented by the papers, patents, and patent applications cited supra deals, in large part, with

the MAGE family of genes, and the unrelated BAGE gene. It has now been found, however, that additional tumor rejection antigen precursors are expressed by cells. These tumor rejection antigen precursors are referred to as "GAGE" tumor rejection antigen precursors. They do not show homology to either the MAGE family of genes or the BAGE gene. Thus the present invention relates to genes encoding such TRAPs, the tumor rejection antigen precursors themselves as well as applications of both.

Thus, another feature of the invention are peptides which are anywhere from 9 to 16 amino acids long, and comprise the sequence:

$Xaa_{(1,2)}$ Trp Xaa Xaa Xaa Xaa Xaa Tyr

(SEQ ID NO: 23)

where Xaa is any amino acid and $Xaa_{(1,2)}$ means that 1 or 2 amino acids may be N-terminal to the Trp residue. These peptides bind to, and/or are processed to peptide which bind to HLA-A29 molecules.

The invention is elaborated upon further in the disclosure which follows.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 sets forth lysis studies using CTL clone 76/6.

Figure 2 shows tumor necrosis factor ("TNF") release assays obtained with various transfectants and controls.

Figure 3 compares lysis induced by cytolytic T lymphocytes of clone CTL 76/6. Peptides of varying length were tested, including SEQ ID NO: 4.

Figure 4 presents an alignment of the cDNAs of the six GAGE genes discussed herein. In the figure, identical regions are surrounded by boxes. Translation initiation sites and stop codons are also indicated. Primers, used in polymerase chain

reaction as described in the examples, are indicated by arrows.

Figure 5 sets forth the alignment of deduced amino acid sequences for the members of the GAGE family. Identical regions are shown by boxes, and the antigenic peptide of SEQ ID NO: 4, is shown.

Figure 6 shows the results obtained when each of the GAGE cDNAs was transfected into COS cells, together with HLA-Cw6 cDNA. Twenty-four hours later, samples of CTL 76/6 were added, and TNF release was measured after twenty-four hours.

Figure 7 compares the stimulation of CTL 22/23 by COS-7 cells, transfected with HLA-A29 cDNA, a MAGE, BAGE, or GAGE sequence, as shown. Control values are provided by MZ2-MEL.43 and COS cells, as stimulators.

Figure 8 presents results obtained from ^{51}Cr release studies, using various peptides including SEQ ID NO: 22 and various peptides derived therefrom.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

Example 1

A melanoma cell line, MZ2-MEL was established from melanoma cells taken from patient MZ2, using standard methodologies. This cell line is described, e.g., in PCT Application PCT/US92/04354, filed May 22, 1992, published November 26, 1992, and incorporated by reference in its entirety. Once the cell line was established, a sample thereof was

irradiated, so as to render it non-proliferative. These irradiated cells were then used to isolate cytolytic T cell clones ("CTLs") specific thereto.

A sample of peripheral blood mononuclear cells ("PBMCs") was taken from patient MZ2, and contacted to the irradiated melanoma cells. The mixture was observed for lysis of the melanoma cells, which indicated that CTLs specific for a complex of peptide and HLA molecule presented by the melanoma cells were present in the sample.

The lysis assay employed was a chromium release assay following Herin et al., Int. J. Cancer 39:390-396 (1987), the disclosure of which is incorporated by reference. The assay, however, is described herein. The target melanoma cells were grown in vitro, and then resuspended at 10^7 cells/ml in DMEM, supplemented with 10 mM HEPES and 30% FCS, and incubated for 45 minutes at 37°C with 200 μCi/ml of $Na(^{51}Cr)O_4$. Labelled cells were washed three times with DMEM, supplemented with 10 mM Hepes. These were then resuspended in DMEM supplemented with 10 mM Hepes and 10% FCS, after which 100 ul aliquots containing 10^3 cells, were distributed into 96 well micro-plates. Samples of PBLs were added in 100 μl of the same medium, and assays were carried out in duplicate. Plates were centrifuged for 4 minutes at 100g, and incubated for four hours at 37°C in an 8% CO_2 atmosphere.

Plates were centrifuged again, and 100 ul aliquots of supernatant were collected and counted. Percentage of ^{51}Cr release was calculated as follows:

$$\% \ ^{51}Cr \text{ release} = \frac{(ER-SR)}{(MR-SR)} \times 100$$

where ER is observed, experimental ^{51}Cr release, SR is spontaneous release measured by incubating 10^3 labeled cells in 200 ul of medium alone, and MR is maximum release, obtained by adding 100 ul 0.3% Triton X-100 to target cells.

Those mononuclear blood samples which showed high CTL activity were expanded and cloned via limiting dilution, and were screened again, using the same methodology. The CTL clone MZ2-CTL 76/6 was thus isolated. The clone is referred to as "76/6" hereafter.

The same method was used to test target K562 cells, as well as the melanoma cell line. Figure 1 shows that this CTL clone recognizes and lyses the melanoma cell line, i.e. MZ2-MEL but not K562. The clone was then tested against other melanoma cell lines and autologous EBV-transformed B cells in the same manner described supra. Figure 1 shows that autologous B cells, transformed by Epstein Barr Virus ("EBV") were not lysed, and that while MZ2-MEL 3.0 was lysed by CTL clone 76/6, the cell line MZ2-MEL.4F$^-$, a variant which does not express antigen F was not. Hence, the clone appears to be specific for this antigen.

The results presented supra are inconclusive as to which HLA molecule presents the TRA. The lysed cell line, i.e., MZ2-MEL, is known to express HLA-A1, HLA-A29, HLA-B37, HLA-B44, HLA-Cw6, and HLA-C clone 10. In experiments not reported here but which follow the protocol of this example, a subline of MZ2-MEL was tested, which had lost expression of

HLA molecules A29, B44, and C clone 10. The subline was lysed, thus indicating that the presenting molecule should be one of A1, B37, or Cw6.

Example 2

Further studies were carried out to determine if 76/6 also produced tumor necrosis factor ("TNF") when contacted with target cells. The method used was that described by Traversari et al., Immunogenetics 35: 145-152 (1992), the disclosure of which is incorporated by reference. Briefly, samples of the CTL line were combined with samples of a target cell of interest in culture medium. After 24 hours, supernatant from the cultures was removed, and then tested on TNF-sensitive WEHI cells. Cell line MZ2-MEL.43, a subclone of the MZ2-MEL cell line discussed supra as well as in the cited references, gave an extremely strong response, and was used in the following experiments.

Example 3

The results from Example 2 indicated that MZ2.MEL.43 presented the target antigen of interest. As such, it was used as a source of total mRNA to prepare a cDNA library.

Total RNA was isolated from the cell line. The mRNA was isolated using an oligo-dT binding kit, following well recognized techniques. Once the mRNA was secured, it was transcribed into cDNA, via reverse transcription, using an oligo dT primer containing a NotI site, followed by second strand synthesis. The cDNA was then ligated to a BstXI adaptor,

digested with NotI, size fractionated on a Sephacryl S-500 HR column, and then cloned, undirectionally, into the BstXI and Not I sites of pcDNA-I-Amp. The recombinant plasmid was then electroporated into DH5α E. coli bacteria. A total of 1500 pools of 100 recombinant bacteria were seeded in microwells. Each contained about 100 cDNAs, because nearly all bacteria contained an insert.

Each pool was amplified to saturation and plasmid DNA was extracted by alkaline lysis and potassium acetate precipitation, without phenol extraction.

Example 4

Following preparation of the library described in Example 3, the cDNA was transfected into eukaryotic cells. The transfections, described herein, were carried out in duplicate. Samples of COS-7 cells were seeded, at 15,000 cells/well into tissue culture flat bottom microwells, in Dulbecco's modified Eagles Medium ("DMEM") supplemented with 10% fetal calf serum. The cells were incubated overnight at 37°C, medium was removed and then replaced by 50 μl/well of DMEM medium containing 10% Nu serum, 400 μg/ml DEAE-dextran, and 100 μM chloroquine, plus 100 ng of the plasmids. As was indicated supra, the lysis studies did not establish which HLA molecule presented the antigen. As a result, cDNA for each of the HLA molecules which could present the antigen (A1, B37, Cw6) was used, separately, to cotransfect the cells. Specifically, one of 28 ng of the gene encoding HLA-A1, cloned into pCD-SRα was used, as were 50 ng of cDNA for

HLA-B37 in pcDNA-I-Amp, or 75 ng of cDNA for HLA-Cw6 in pcDNAI/Amp, using the same protocol as was used for transfection with the library.

Transfection was made in duplicate wells, but only 500 pools of the HLA-Cw6 transfectants could be tested in single wells. Following four hours of incubation at 37°C, the medium was removed, and replaced by 50 μl of PBS containing 10% DMSO. This medium was removed after two minutes and replaced by 200 μl of DMEM supplemented with 10% FCS.

Following this change in medium, COS cells were incubated for 24-48 hours at 37°C. Medium was then discarded, and 1000-3000 cells of CTL clone 76/6 were added, in 100 μl of Iscove's medium containing 10% pooled human serum supplemented with 20-30 U/ml of IL-2. Supernatant was removed after 24 hours, and TNF content was determined in an assay on WEHI cells, as described by Traversari et al., Immunogenetics 35: 145-152 (1992), the disclosure of which is incorporated by reference.

The 1500 pools transfected with HLA-A1, and the 1500 pools transfected with HLA-B37 stimulated TNF release to a concentration of 15-20 pg/ml, or 2-6 pg/ml, respectively. Most of the HLA-Cw6 transfectants yielded 3-20 pg/ml, except for one pool, which yielded more than 60 pg/ml. This pool was selected for further work.

Example 5

The bacteria of the selected pool were cloned, and 600 clones were tested. Plasmid DNA was extracted therefrom,

transfected into a new sample of COS cells in the same manner as described supra, and the cells were again tested for stimulation of CTL clone 76/6. Ninety-four positive clones were found. One of these, referred to as cDNA clone 2D6, was tested further. In a comparative test COS cells were transfected with cDNA clone 2D6 and the HLA-Cw6 cDNA, HLA-Cw6 cDNA alone, or cDNA 2D6 alone. Control cell lines MZ2-MEL F^- and MZ2-MEL F^+ were also used. TNF release into CTL supernatant was measured by testing it on WEHI cells, as referred to supra. The number of surviving WEHI cells was measured by optical density after incubation of the cells with MTT. Figure 2 shows that the COS cells transfected with HLA-Cw6 and cDNA-2D6, and the cell line MZ2-MEL F^+ stimulated TNF release from CTL clone 76/6, indicating that HLA-Cw6 presented the subject TRA.

Example 6

The cDNA 2D6 was sequenced following art known techniques. A sequence search revealed that the plasmid insert showed no homology to known genes or proteins. SEQ ID NO: 1 presents cDNA nucleotide information for the identified gene, referred to hereafter as "GAGE". A putative open reading frame is located at bases 51-467 of the molecule. The first two bases of this sequence are from the vector carrying the cDNA sequence, and are thus not part of the cDNA itself.

Example 7

Following sequencing of the cDNA, as per Example 6, experiments were carried out to determine if cells of normal tissues expressed the gene. To determine this, Northern blotting was carried out on tissues and tumor cell lines, as indicated below. The blotting experiments used cDNA for the complete sequence of SEQ ID NO: 1. PCR was then used to confirm the results.

Table 1. Expression of gene GAGE

Normal tissues	
PHA activated T cells	-
CTL clone 82/30	-
Liver	-
Muscle	-
Lung	-
Brain	-
Kidney	-
Placenta	-
Heart	-
Skin	-
Testis	+
Tumor cell lines	
Melanoma	7/16
Lung Carcinoma	1/6
Sarcoma	0/1
Thyroid medullary carcinoma	0/1
Tumor samples	
Melanoma	1/1

Example 8

Detailed analysis of normal tissues and tumors was carried out by applying polymerase chain reaction ("PCR") and the GAGE gene information described supra.

First, total RNA was taken from the particular sample, using art recognized techniques. This was used to prepare cDNA. The protocol used to make the cDNA involved combining 4 ul of reverse transcriptase buffer 5x, 1 ul of each dNTP, (10 mM), 2 ul of dithiothreitol (100 mM), 2 ul of dT-15 primer (20 um), 0.5 ul of RNasin (40 units/ul), and 1 ul of MoMLV reverse transcriptase (200 units/ul). Next, 6.5 ul of template RNA (1 ug/3.25 ul water, or 2 ug total template RNA) was added. The total volume of the mixture was 20 ul. This was mixed and incubated at 42°C for 60 minutes, after which it was chilled on ice. A total of 80 ul of water was then added, to 100 ul total. This mixture was stored at -20°C until used in PCR.

To carry out PCR, the primers

5'-AGA CGC TAC GTA GAG CCT-3'

(sense)

and

5'-CCA TCA GGA CCA TCT TCA-3'

(antisense)

SEQ ID NOS: 2 and 3, respectively, were used. The reagents included 30.5 ul water, 5 ul of PCR buffer 10x, 1 ul of each dNTP (10 uM), 2.5 ul of each primer (20 uM), and 0.5 ul of polymerizing enzyme "Dynazyme (2 units/ul). The total volume was 45 ul. A total of 5 ul of cDNA was added (this corre-

sponded to 100 ng total RNA). The mixture was combined, and layered with one drop of mineral oil. The mixture was transferred to a thermocycler block, preheated to 94°C, and amplification was carried out for 30 cycles, each cycle consisting of the following:

first denaturation:	94°C, 4 min.
denaturation:	94°C, 1 min.
annealing:	55°C, 2 min.
extension:	72°C, 3 min.
final extension:	72°C, 15 min.

Following the cycling, 10 ul aliquots were run on a 1.5% agarose gel, stained with ethidium bromide.

cDNA amplified using the primers set forth supra yields a 238 base pair fragment. There is no amplification of contaminating genomic DNA, if present.

The results are presented in Table 2, which follows. They confirm that the only normal tissue which expresses GAGE is testis, whereas a number of tumors, including melanoma, lung, breast, larynx, pharynx, sarcoma, testicular seminoma, bladder and colon express the gene. Thus, any one of these tumors can be assayed for by assaying for expression of the GAGE gene.

Table 2

RT-PCR analysis of the expression of gene GAGE

NORMAL TISSUES	
Heart	-
Brain	-
Liver	-
Lung	-
Kidney	-
Spleen	-
Lymphocytes	-
Bone marrow	-
Skin	-
Naevus	-
Melanocytes	-
Fibroblasts	-
Prostate	-
Testis	+
Ovary	-
Breast	-
Adrenals	-
Muscle	-
Placenta	-
Umbilical Cord	-

TUMORS	Cell lines	Tumor samples	
Melanoma	40/63	46/146	(32%)
Lung cancer			
Epidermoid carcinoma		10/41	(24%)
Adenocarcinoma		4/18	
Small Cell Lung Cancer	6/23	0/2	
Breast cancer		15/146	(10%)
Head and Neck tumor			
Larynx		6/15	(40%)
Pharynx		3/13	
Sarcoma	1/4	6/18	(33%)
Testicular seminoma		6/6	(100%)
Bladder cancer		5/37	(14%)
Prostate cancer		2/20	
Colon carcinoma	5/13	0/38	
Renal cancer	0/6	0/45	
Leukemia	3/6	0/19	

Example 9

The identification of the nucleic acid molecule referred to in the prior examples led to further work directed to the determination of tumor rejection antigens presented by HLA-Cw6 molecules, and derived from the GAGE gene.

The complete cDNA of GAGE in expression vector pcDNAI/Amp was digested with restriction endonucleases NotI and SpHI, and then with exonuclease III following supplier's instruction (Erase-a-base System, Promega). This treatment generated a series of progressive deletions, starting at the 3'end.

The deletion products were ligated back into pcDNAI/Amp, and then electroporated into E. coli strain DH5α'IQ, using well known techniques. The transformants were selected with ampicillin (50 micrograms/ml).

Plasmid DNA was extracted from each recombinant clone and was then transfected into COS-7 cells, together with a vector which coded for HLA-Cw6. The protocols used follow the protocols described above.

The transfectants were then tested in the TNF release assay. This permitted separation of positive and negative clones. All the negative clones showed a deletion of the entire GAGE sequence. The smallest positive clone contained the first 170 nucleotides of SEQ ID NO: 1. The analysis of this sequence, supra, notes that the open reading frame starts at nucleotide 51. Thus, this fragment contains a sequence which encodes the first 40 amino acids of the GAGE TRAP.

Example 10

Additional experiments were then carried out to define the region encoding the TRA peptide more precisely. Polymerase chain reaction ("PCR") amplification was used to do this.

Two primers were synthesized. The first primer was a 22-mer complementary to a sequence within the plasmid vector pcDNAI/Amp located upstream of a BamHI site. The second primer was a 29-mer containing at the 3'end nucleotides 102-119 of SEQ ID NO: 1, and at the 5'end an extension of 11 nucleotides containing an XbaI restriction site.

Following amplification, the PCR product was digested by BamHI and XbaI, and cloned into the BamHI-XbaI sites of plasmid pcDNA-3. The recombinant colonies were cotransfected into COS-7 cells with cDNA encoding HLA-Cw6, in accordance with Example 4, and a TNF release assay, also as described supra, was carried out, using CTL 76/6.

TNF release was observed, indicating that the "minigene" was processed to a TRA. The minigene, i.e., nucleotides 1-119 of SEQ ID NO: 1, the coding region of which runs from nucleotides 51-119 encoded the first 23 amino acids of the cDNA of SEQ ID NO: 1. This information served as the basis for the next set of experiments.

Example 11

Two peptides were synthesized, based upon the first 23 amino acids of SEQ ID NO: 1. These were:

Met Ser Trp Arg Gly Arg Ser Thr Tyr Arg Pro Arg Pro Arg Arg
(SEQ ID NO: 2)

and

Thr Tyr Arg Pro Arg Pro Arg Arg Tyr Val Glu Pro Pro Glu Met Ile
(SEQ ID NO: 3)

Each peptide was pulsed into COS-7 cells previously transfected with HLA-Cw6 cDNA, and combined with CTL 76/6 to determine if TNF release would be induced. Peptides (20 ug/ml) were added to COS-7 cells which had been transfected with the HLA-Cw6 cDNA twenty-four hours previously. After incubation at 37°C for 90 minutes, medium was discarded, and 3000 CTLs were added in 100 microliters of medium, containing 25 units/ml of IL-2. Eighteen hours later, TNF content of supernatant was tested via determining toxicity on WEHI-164-13 cells. The second peptide (SEQ ID NO: 3) was found to induce more than 30 pg/ml of TNF, while the first peptide (SEQ ID NO: 2), was found to induce less than 10 pg/ml of TNF. The second peptide was used for further experiments.

Example 12

Various peptides based upon SEQ ID NO: 3 were synthesized, and tested, some of which are presented below. To carry out these tests, ^{51}Cr labelled LB33-EBV cells, which are HLA-Cw6 positive, were incubated with one of the following peptides:

Tyr Arg Pro Arg Pro Arg Arg Tyr
(SEQ ID NO: 4)

Thr Tyr Arg Pro Arg Pro Arg Arg Tyr
(SEQ ID NO: 5)

Tyr Arg Pro Arg Pro Arg Arg Tyr Val
(SEQ ID NO: 6)

Thr Tyr Arg Pro Arg Pro Arg Arg Tyr Val
(SEQ ID NO: 7)

Arg Pro Arg Pro Arg Arg Tyr Val Glu
(SEQ ID NO: 8)

Met Ser Trp Arg Gly Arg Ser Thr Tyr Arg Pro Arg Pro Arg Arg
(SEQ ID NO: 2)

The peptide concentration varied, as indicated in figure 3, and the ratio of CTL: LB33-EBV ("effector: target ratio"), was 10:1. ^{51}Cr release was determined after four hours of incubation at 37°C. Levels of lysis for positive ("F+", MZ2-MEL.3.1), and negative ("F-"; MZ2-MEL.2.2.5) control cells are indicated, in figure 3.

It was found, quite surprisingly, that the octamer of SEQ ID NO: 4 was the best peptide, and appeared to be the tumor rejection antigen. This is the first time an octamer has been reported as being involved in presentation by a human MHC molecule. There is some precedent for a murine system, as reported by Engelhard, supra, at 199, for H-2K^b and H-2K^k molecules. The nonamers of SEQ ID NO: 5 and SEQ ID NO: 6 also induced CTL lysis albeit to a lesser extent than the octamer of SEQ ID NO: 4.

In results not reported here, a second CTL was tested (CTL 82/31). This CTL was known to lyse cells presenting MZ2-F. It, too, lysed HLA-Cw6 positive cells following pulsing with the peptide of SEQ ID NO: 4.

Example 13

To find out whether the GAGE DNA set forth supra was unique, a cDNA library made with RNA from MZ2-MEL.43 (the same library that was used for the cloning of GAGE) was hybridized with a probe derived from the GAGE cDNA. The probe was a PCR fragment of 308 base pairs between positions 20 and 328 of SEQ ID NO: 1. Twenty positive cDNAs were obtained. Six of them were entirely sequenced. They were all highly related to the GAGE sequence, but they were slightly different from it. Two of the six clones were identical to each other, but all the others differed from each other. Thus, five new sequences different from but highly related to GAGE were identified. They are called GAGE-2, 3, 4, 5 and 6 (Figure 4). The fourteen other clones were partially sequenced at the 5' end and their sequence corresponded to one of the six GAGE cDNAs.

The major difference between these cDNAs and GAGE-1 is the absence of a stretch of 143 bases located at position 379 to 521 of the GAGE sequence of SEQ ID NO: 1. The rest of the sequences shows mismatches only at 19 different positions, with the exception of GAGE-3 whose 5'end is totally different from the other GAGE for the first 112 bases. This region of the GAGE-3 cDNA contains a long repeat and a hairpin structure.

The deduced GAGE-1 protein corresponding to a tumor rejection antigen precursor is about 20 amino acids longer than the 5 other proteins, whose last seven residues also differ from the homologous residues of GAGE-1 (Figure 5).

The rest of the protein sequences show only 10 mismatches. One of these is in the region corresponding to the antigenic peptide of SEQ ID NO: 4. The sequence of the peptide is modified in GAGE-3, 4, 5 and 6 so that position 2 is now W instead of R.

Example 14

To assess whether the change at position 2 affected the antigenicity of the peptide, cDNA of the 6 GAGE cDNAs were individually transfected into COS cells together with the cDNA of HLA-Cw6, and the transfectants were tested for recognition by CTL 76/6 as described, supra. Only GAGE-1 and GAGE-2 transfected cells were recognized, showing that the modified peptide encoded by GAGE-3, 4, 5 and 6 was not antigenic in the context of this experiment. Sequence analysis of the 5' end of the 14 other clones mentioned supra, showed that 7 of them contained the sequence encoding the antigenic peptide, and thus probably corresponded to either GAGE-1 or GAGE-2.

Example 15

The PCR primers used, supra to test the expression of GAGE in tumor samples do not discriminate between GAGE-1 or 2 and the four other GAGE cDNAs that do not encode antigen MZ2F. A new set of primers was prepared which specifically amplifies GAGE-1 and 2, and not GAGE-3, 4, 5 and 6. These primers are:

VDE44 5'-GAC CAA GAC GCT ACG TAG-3' (SEQ ID NO: 9)
VDE24 5'-CCA TCA GGA CCA TCT TCA-3' (SEQ ID NO: 10)

These primers were used as described, supra, in a RT-PCR reaction using a polymerase enzyme in the following temperature conditions:

4 min at 94°C
30 cycles with 1 min at 94°C
2 min at 56°C
3 min at 72°C
15 min at 72°C

The results of this analysis are set forth in Table 3.

Table 3

Expression of GAGE genes by tumor samples and tumor cell lines

Histological type	Number of GAGE positive tumors	
	All GAGE genes*	GAGE-1 and 2**
Tumor samples		
Melanomas		
primary lesions	5/39	5/39 (13%)
metastases	47/132	36/131 (27%)
Sarcomas	6/20	6/20 (30%)
Lung carcinomas NSCLC	14/65	12/64 (19%)
Head and neck squamous cell carcinomas	13/55	10/54 (18%)
Prostatic carcinomas	2/20	2/20
Mammary carcinomas	18/162	14/162 (9%)
Bladder carcinomas		
superficial	1/20	1/20
infiltrating	3/26	3/26
Testicular seminomas	6/6	5/6
Colorectal carcinomas	0/43	
Leukemias and lymphomas	0/25	
Renal carcinomas	0/46	
Tumor cell lines		
Melanomas	45/74	40/74 (54%)
Sarcomas	1/4	1/4
Lung carcinomas		
SCLC	7/24	7/24 (29%)
NSCLC	1/2	1/2
Mesotheliomas	5/19	5/19 (26%)
Head and neck squamous cell carcinomas	0/2	
Mammary carcinomas	1/4	0/4
Bladder carcinomas	0/3	
Colon carcinomas	3/13	3/13
Leukemias	3/6	1/6
Lymphomas	0/6	
Renal carcinomas	0/6	

* Expression of GAGE was tested by RT-PCR on total RNA with primers VDE-18 and VDE-24, detecting all GAGE genes. No PCR product was observed when these primers were assayed on DNA from MZ2-MEL.
** Expression of GAGE-1 and 2 was tested by RT-PCR on total RNA with primers VDE-44 and VDE-24, which distinguish GAGE-1 and 2 from the four other GAGE genes. No PCR product was observed when these primers were assayed on DNA from MZ2-MEL.

In further work, new primers were designed which amplified all GAGE genes, to make sure that there was no expression of any of them in normal tissues. These primers are

VDE43 5'-GCG GCC CGA GCA GTT CA-3' (SEQ ID NO: 11)
VDE24 5'-CCA TCA GGA CCA TCT TCA-3 (SEQ ID NO: 10)

These were used exactly as for the PCR using the VDE44 and VDE24 primers. The results are shown in Table 4. They confirm that the normal tissues are negative, except for testis.

Table 4

Expression of GAGE genes in normal adult and fetal tissues

Adult tissues	GAGE expression*
Adrenal gland	-
Benign naevus	-
Bone marrow	-
Brain	-
Breast	-
Cerebellum	-
Colon	-
Heart	-
Kidney	-
Liver	-
Lung	-
Melanocytes	-
Muscle	-
Ovary	-
Prostate	-
Skin	-
Splenocytes	-
Stomach	-
Testis	+
Thymocytes	-
Urinal bladder	-
Uterus	-
Placenta	-
Umbilical cord	-
Fetal tissues†	
Fibroblasts	-
Brain	-
Liver	-
Spleen	-
Thymus	-
Testis	+

*Expression of GAGE was tested by RT-PCR amplification on total RNA with primers VDE43 and VDE24 detecting all GAGE genes (Figure 7). Absence of PCR product is indicated by - and presence by +. No PCR product was observed when these primers were assayed on DNA from MZ2-MEL.

†Fetal tissues derive from fetuses older than 20 weeks.

Example 16

In work not reported here, it had been ascertained that cytolytic T cell clone CTL 22/23 (Van den Eynde, et al., Int. J. Cancer 44: 634-640 (1989), incorporated by reference) did not recognize melanoma cell line MZ2-MEL.3.1. This melanoma cell line was reported by Van der Bruggen, et al., Eur. J. Immunol. 24: 2134-2140 (1994), to have lost expression of MHC molecules HLA-A29, HLA-B24, and HLA-cw·1601. Studies were undertaken to determine if transfection with one of these MHC molecules could render the line sensitive to CTL 22/23. HLA-A29 was the first molecule tested. To do so, poly A' RNa was extracted from HLA-A29' cell line MZ2-MEL.43, using a commercially available extraction kit, and following the manufacturer's instructions. The mRNA was then converted to cDNA, using standard methodologies, size fractionated, and then inserted unidirectionally, into the Bstx1 and NotI sites of plasmid pcDNA-I/Amp. The plasmids were electroporated into E. coli strain DH5α'IQ, and selected with ampicillin (50 μg/ml). The bacteria were plated onto nitrocellulose filters, and duplicated. The filters were prepared, and hybridized overnight in 6xSSC/0.1% SDS/1x Denhardt's solution at 40°C, using ^{32}P labelled probe:

5'ACTCCATGAGGTATTTC-3'

(SEQ ID NO: 19)

The probe is a sequence which surrounds the start codon of HLA sequences.

The filters were washed twice, at room temperature for 5 minutes each time in 6xSSC, and twice in 6xSSC at 43°C. Positive sequences were then screened with probe:

5'-TTTCACCACATCCGTGT-3'

(SEQ ID NO: 20)

which had been labelled with ^{32}P. This sequence is specific for HLA-A29, as determined by reference to the Kabat Database of sequences and proteins of immunological interest, incorporated by reference. This database is available at the NCBI (USA), or on Web Sotle (Internet) WWW.NCBI.NLM.NIH.GOV. Teh filters were washed twice at room temperature for 5 minutes each time, at 6xSSC, followed by two washes, at 6xSSC (5 minutes per wash), at 42°C.

Example 17

Once positive HLA-A29 clones were isolated, these were transfected into COS-7 using the DEAE-dextran chloroquine method set out supra. In brief, 1.5 x 10^4 COS-7 cells were treated with 50ng of plasmid pcDNA-I/Amp containing HLA-A29, and 100 ng of cDNA containing cDNA for one of the GAGE sequences mentioned supra, or one of the prior art MAGE or BAGE sequences in plasmid pcDNAα-I/Amp or pcDSRα-respectively. The transfectants were then incubated for 24 hours at 37°C.

The transfectants were then tested for their ability to stimulate TNF production by CTLs, using the assay explained at the end of example 4, supra.

Figure 7, which presents the results of this drug, shows that high levels of TNF production were achieved using any of

GAGE-3, 4, 5 or 6 and HLA-A29 as transfectants. GAGE-1 and GAGE-2, in contrast, do not stimulate CTL clone 22/23, thus leading to the conclusion that GAGE 3, 4, 5 and 6 are processed to an antigen or antigens presented by HLA-A29 molecules and recognized by CTL 22/23.

Example 18

The fact that GAGE-3, 4, 5 and 6 were processed to peptides presented by HLA-A29' cells, which GAGE-1 and GAGE-2 were not, suggested examination of the deduced amino acid sequences for those common to GAGE 3, 4, 5 and 6 and absent from GAGE-1 and GAGE-2.

The sequence:

Arg Ser Thr Tyr Tyr Trp Pro Arg Pro Arg Arg Tyr Val Gln

(SEQ ID NO: 21)

was identified. The peptide was synthesized, lyophilized, and then dissolved in 1 volume DMSO, 9 volumes of 10 mM acetic acid in water. This methodology was used for the other peptides synthesized, discussed infra.

The peptide (SEQ ID NO: 21) was tested in a ^{51}Cr release experiment, following the method described supra.

It was found that this peptide did provoke lysis. Successive deletions were prepared, and tested for their ability to provoke lysis, again using the ^{51}Cr lytic assay. This work is depicted in Figure 8. It was found that the shortest peptide to provoke lysis was

Tyr Tyr Trp Pro Arg Pro Arg Arg Tyr

(SEQ ID NO: 22), which is common to all of GAGE-3 through 6.

Specifically, amino acids 10-18 of GAGE-3, and amino acids 9-17 of GAGE-4, 5 and 6 correspond to this peptide.

The members of the peptide family shown in Figure 8, and represented, e.g., by SEQ ID NOS: 21 and 22, do not accord with the data presented by Toubert, et al., "HLA-A29 Peptide Binding Motif", Abstract No. 4183, Ninth International Congress of Immunology, July 23-29, 1995, San Francisco, CA, incorporated by reference. According to Toubert, et al., at the least a Phe residue is required at the third position of any peptide which binds to HLA-A29. As is shown herein, such is not the case.

Example 19

A set of experiments were carried out to isolate and to clone genomic DNA sequences encoding GAGE TRAPS.

A library was made from genomic DNA isolated from the peripheral blood lymphocytes of patient MZ2. Isolation and preparation of the DNA was carried out in accordance with Wölfel et al., Immunogenetics 26: 178-187 (1987), incorporated by reference. The isolated DNA was then partially digested with the restriction enzyme Sau3A, and then fractionated using NaCl density gradient ultracentrifugation. This provides a fraction enriched in 10-20 kb fragments of DNA. See Grosveld et al., Nucl. Acids. Res. 10: 6715-6732 (1982). These fragments were dephosphorylated using alkaline phosphatase, and were then ligated into λ-Gem11 DNA, which had been digested with BamHI/EcoRI. Briefly, 2 ugs of the genomic DNA were mixed with 2 ugs of the λ phage DNA in a 10

ul volume, and incubated at 16°C overnight. 4 μl of the ligation mixture containing the ligated DNA was packaged, in vitro, in a commercially available phage packaging extract. The resulting phages were titrated on E. coli NM539 (a commercially available strain), in order to calculate the appropriate number of phages to plate out for screening. The resulting product was titrated onto cells of E. coli strain NM539.

Example 20

Approximately 33,333 recombinant phages were plated per plate, to give a total of 500,000 phages tested. A total of 20 μl of the packaging mixture was mixed with 1 ml of a suspension of E. coli NM539 in 10 mM $MgSO_4$, to an OD_{600} of 0.5. This mixture was then incubated, for 15 minutes at 37°C, and then mixed with 15 ml of culture medium BTCYM containing 0.7% agarose at 45°C, and then plated onto agar plates containing BTCYM. The resulting mixture was incubated, at 37°C, overnight. The resulting phage plaques were used in hybridization experiments. Approximately 500,000 recombinant phage plaques were immobilized on nylon membranes, and were then subjected to in situ hybridization, in accordance with Sambrook et al., Molecular Cloning: A Laboratory Manual (1989), incorporated by reference.

The hybridization was carried out using a probe which consisted of nucleotides 18 through 326 of SEQ ID NO: 1. The probe was prepared using the polymerase chain reaction and, as primers, a nucleotide sequence consisting of nucleotides

18-34 and the complement of nucleotides 309-326 of this sequence. The primers were used in a 30 cycle PCR run (1 cycle: 94°C for one minute, followed by 46°C for two minutes, then 72°C for three minutes), in a total volume of 100 ul, which contained 10 ul of 10x concentrated Dynazyme buffer, 0.2 mM of each dNTP, 50 pmoles of each primer, and 2.5 units of Dynazyme DNA polymerase.

The probe was then purified via electrophoresis in low temperature melting agarose, as described by Sambrook et al., supra. Following purification, the probe was radiolabelled with $\alpha^{32}P$, using a commercially available, random priming kit (radioactive nucleotide was $\alpha^{32}P$ dCTP).

Once the probes were labelled, they were used in a hybridization buffer (10% sodium salt of dextran sulfate, MW 500,000; 1% SDS; 1M NaCl, and 50 ug/ml of denatured salmon sperm DNA). About 150 ng of ^{32}P labelled probe (approximately $1.6x10^8$cpm), were put into a total volume of 200 ml of this buffer. Approximately 500,000 immobilized plaques on filters were hybridized filter which was combined with the nylon membrane containing at 65°C for about 15 hours. The membranes were then washed with 0.2xSSC, 0.1% SDS, at 65°C.

Following autoradiography, one positive clone was found. When excised, the insert was found to be about 11 kilobases long. Three fragments (175 base pairs, 4.5 kilobases, and 6.5 kilobases) resulted from treatment of the insert with the endonuclease SstI, and these were then subcloned into the plasmids pBluescript SK(-), and pTZ19R, both of which are commercially available. The fragments were sequenced in

their entirety, using commercially available enzymes, and primers 5'-labelled with [γ^{33}P]ATP. The sequence of the genomic clone is provided as SEQ ID NO: 24.

The foregoing examples show the isolation of nucleic acid molecules which code for tumor rejection antigen precursors and tumor rejection antigens. These molecules, however, are not homologous with any of the previously disclosed MAGE and BAGE coding sequences described in the references set forth supra. Hence, one aspect of the invention is an isolated nucleic acid molecule which comprises the nucleotide sequence set forth in SEQ

ID NO: 1 as well as fragments thereof, such as nucleotides 1-170, and 51-170, and any other fragment which is processed to a tumor rejection antigen. The sequence of SEQ ID NO: 1 is neither a MAGE nor a BAGE coding sequence, as will be seen by comparing it to the sequence of any of these genes as described in the cited references. Also a part of the invention are those nucleic acid molecules which also code for a non-MAGE and non-BAGE tumor rejection antigen precursor but which hybridize to a nucleic acid molecule containing the described nucleotide sequence, under stringent conditions. The term "stringent conditions" as used herein refers to parameters with which the art is familiar. More specifically, stringent conditions, as used herein, refers to hybridization in 1M NaCl, 1% SDS, and 10% dextran sulfate. This is followed by two washes of the filter at room temperature for 5 minutes, in 2xSSC, and one wash for 30 minutes in 2xSSC, 0.1% SDS. There are other conditions, reagents, and

so forth which can be used, which result in the same or higher degree of stringency. The skilled artisan will be familiar with such conditions, and, thus, they are not given here.

It will also be seen from the examples that the invention embraces the use of the sequences in expression vectors, as well as to transform or transfect host cells and cell lines, be these prokaryotic (e.g., E. coli), or eukaryotic (e.g., CHO or COS cells). The expression vectors require that the pertinent sequence, i.e., those described supra, be operably linked to a promoter. As it has been found that human leukocyte antigen HLA-Cw6 presents a tumor rejection antigen derived from these genes, the expression vector may also include a nucleic acid molecule coding for HLA-Cw6. In a situation where the vector contains both coding sequences, it can be used to transfect a cell which does not normally express either one. The tumor rejection antigen precursor coding sequence may be used alone, when, e.g., the host cell already expresses HLA-Cw6. Of course, there is no limit on the particular host cell which can be used. As the vectors which contain the two coding sequences may be used in HLA-Cw6 presenting cells if desired, and the gene for tumor rejection antigen precursor can be used in host cells which do not express HLA-Cw6.

The invention also embraces so called expression kits, which allow the artisan to prepare a desired expression vector or vectors. Such expression kits include at least separate portions of each of the previously discussed coding

sequences. Other components may be added, as desired, as long as the previously mentioned sequences, which are required, are included.

To distinguish the nucleic acid molecules and the TRAPs of the invention from the previously described MAGE and BAGE materials, the invention shall be referred to as the GAGE family of genes and TRAPs. Hence, whenever "GAGE" is used herein, it refers to the tumor rejection antigen precursors coded for by the previously described sequences. "GAGE coding molecule" and similar terms, are used to describe the nucleic acid molecules themselves.

The invention as described herein has a number of uses, some of which are described herein. First, the invention permits the artisan to diagnose a disorder such as melanoma, characterized by expression of the TRAP, or presentation of the tumor rejection antigen. These methods involve determining expression of the TRAP gene, and/or TRAs derived therefrom, such as a TRA presented by HLA-Cw6. In the former situation, such determinations can be carried out via any standard nucleic acid determination assay, including the polymerase chain reaction, or assaying with labelled hybridization probes. In the latter situation, assaying with binding partners for complexes of TRA and HLA, such as antibodies, is especially preferred. An alternate method for determination is a TNF release assay, of the type described supra. To carry out the assay, it is preferred to make sure that testis cells are not present, as these normally express GAGE. This is not essential, however, as one can routinely

differentiate between testis and other cell types. Also, it is practically impossible to have testis cells present in non-testicular sample.

The isolation of the TRAP gene also makes it possible to isolate the TRAP molecule itself, especially TRAP molecules containing the amino acid sequence coded for by SEQ ID NOS: 2-6. These isolated molecules when presented as the TRA, or as complexes of TRA and HLA, such as HLA-Cw6 or HLA-A29 may be combined with materials such as adjuvants to produce vaccines useful in treating disorders characterized by expression of the TRAP molecule.

Exemplary adjuvants include Freund's complete and incomplete adjuvant, killed B. pertussis organism, "BCG", or Bacille Calmente-Guerin, $Al(OH)_3$, muramyl dipeptide and its derivatives which may be emulsified in metabolizable oils, such as squalene, monophosphoryl lipid A (MPL), keyhold limpet hemocyanin (KLH), saponin extracts such as QA-7, QA-19, and QA-21 (also referred to as QS-21), these having been described in U.S. Patent No. 5,057,540 to Kensil, et al., incorporated by reference, MTP-MF59, N-[1-(2,3-dioleoyloxy) propyl]-N,N,N-trimethylammonium methyl sulfate (DOTAP), the cationic amphiphile DOTMA, the neutral phospholipids such as DOPE, and combinations of these. This listing is by no means comprehensive, and the artisan of ordinary skill will be able to augment this listing. All additional adjuvants are encompassed herein.

In addition, vaccines can be prepared from cells which present the TRA/HLA complexes on their surface, such as non-

proliferative cancer cells, non-proliferative transfectants, etcetera. In all cases where cells are used as a vaccine, these can be cells transfected with coding sequences for one or both of the components necessary to provide a CTL response, or be cells which express both molecules without transfection. Further, the TRAP molecule, its associated TRAs, as well as complexes of TRA and HLA, may be used to produce antibodies, using standard techniques well known to the art.

When "disorder" is used herein, it refers to any pathological condition where the tumor rejection antigen precursor is expressed. An example of such a disorder is cancer, melanoma in particular. Melanoma is well known as a cancer of pigment producing cells.

As indicated, supra, tumor rejection antigens, such as the one presented in SEQ ID NO: 4 are also a part of the invention. Also a part of the invention are polypeptides, such as molecules containing from 8 to 16 amino acids, where the polypeptides contain the amino acid sequence set forth in SEQ ID NO: 4. As the examples indicate, those peptides which are longer than the octamer of SEQ ID NO: 4 are processed into the tumor rejection antigen of SEQ ID NO: 4 by the HLA-Cw6 presenting cancer cells, and presented thereby. The presentation leads to lysis by cytolytic T lymphocytes present in a body fluid sample contacted to the cells presenting the complex. Similarly, the peptides longer than SEQ ID NO: 22, such as SEQ ID NO: 21, are processed to the appropriate TRA, and are presented by cancer cells, such as HLA-A29

positive cells.

Thus, another feature of the invention are peptides which are anywhere from 9 to 16 amino acids long, and comprise the sequence:

Xaa Xaa Trp Xaa Xaa Xaa Xaa Xaa Trp

(SEQ ID NO: 23)

where Xaa is any amino acid. These peptides bend to, and/or are processed to peptides which bind to HLA-A29 molecules. The fact that these peptides are processed to the tumor rejection antigen, is indicated by the examples.

This property may be exploited in the context of other parameters in confirming diagnosis of pathological conditions, such as cancer, melanoma in particular. For example, the investigator may study antigens shed into blood or urine, observe physiological changes, and then confirm a diagnosis of melanoma using the CTL proliferation methodologies described herein.

On their own, peptides in accordance with the invention may be used to carry out HLA-typing assays. It is well known that when a skin graft, organ transplant, etc., is necessary one must perform HLA typing so as to minimize the possibility of graft rejection. The peptides of the invention may be used to determine whether or not an individual is HLA-Cw6 positive, so that appropriate donors may be selected. This type of assay is simple to carry out. The peptides of the invention are contacted to a sample of interest, and binding to cells in that sample indicates whether or not the individual from which the sample is taken is HLA-Cw6 positive. One

may label the peptides themselves, conjugate or otherwise bind them to linkers which are labeled, immobilize them to solid phases, and so forth, so as to optimize such an assay. Other standard methodologies will be clear to the skilled artisan, and need not be presented herein.

Therapeutic approaches based upon the disclosure are premised on a response by a subject's immune system, leading to lysis of TRA presenting cells, such as HLA-Cw6 cells. One such approach is the administration of CTLs specific to the complex to a subject with abnormal cells of the phenotype at issue. It is within the skill of the artisan to develop such CTLs in vitro. Specifically, a sample of cells, such as blood cells, are contacted to a cell presenting the complex and capable of provoking a specific CTL to proliferate. The target cell can be a transfectant, such as a COS cell of the type described supra. These transfectants present the desired complex on their surface and, when combined with a CTL of interest, stimulate its proliferation. COS cells, such as those used herein are widely available, as are other suitable host cells.

To detail the therapeutic methodology, referred to as adoptive transfer (Greenberg, J. Immunol. 136(5): 1917 (1986); Riddel et al., Science 257: 238 (7-10-92); Lynch et al., Eur. J. Immunol. 21: 1403-1410 (1991); Kast et al., Cell 59: 603-614 (11-17-89)), cells presenting the desired complex are combined with CTLs leading to proliferation of the CTLs specific thereto. The proliferated CTLs are then administered to a subject with a cellular abnormality which is

characterized by certain of the abnormal cells presenting the particular complex, where the complex contains the pertinent HLA molecule. The CTLs then lyse the abnormal cells, thereby achieving the desired therapeutic goal.

The foregoing therapy assumes that at least some of the subject's abnormal cells present the relevant HLA/TRA complex. This can be determined very easily, as the art is very familiar with methods for identifying cells which present a particular HLA molecule, as well as how to identify cells expressing RNA of the pertinent sequences, in this case a GAGE sequence. Once cells presenting the relevant complex are identified via the foregoing screening methodology, they can be combined with a sample from a patient, where the sample contains CTLs. If the complex presenting cells are lysed by the mixed CTL sample, then it can be assumed that a GAGE derived, tumor rejection antigen is being presented, and the subject is an appropriate candidate for the therapeutic approaches set forth supra.

Adoptive transfer is not the only form of therapy that is available in accordance with the invention. CTLs can also be provoked in vivo, using a number of approaches. One approach, i.e., the use of non-proliferative cells expressing the complex, has been elaborated upon supra. The cells used in this approach may be those that normally express the complex, such as irradiated melanoma cells or cells transfected with one or both of the genes necessary for presentation of the complex. Chen et al., Proc. Natl. Acad. Sci. USA 88: 110-114 (January, 1991) exemplifies this approach, show-

ing the use of transfected cells expressing HPV E7 peptides in a therapeutic regime. Various cell types may be used. Similarly, vectors carrying one or both of the genes of interest may be used. Viral or bacterial vectors are especially preferred. In these systems, the gene of interest is carried by, e.g., a Vaccinia virus or the bacteria BCG, and the materials de facto "infect" host cells. The cells which result present the complex of interest, and are recognized by autologous CTLs, which then proliferate. A similar effect can be achieved by combining the tumor rejection antigen or the precursor itself with an adjuvant to facilitate incorporation into HLA-Cw6 presenting cells which then present the HLA/peptide complex of interest. The TRAP is processed to yield the peptide partner of the HLA molecule while the TRA is presented without the need for further processing.

Other aspects of the invention will be clear to the skilled artisan and need not be repeated here.

The terms and expressions which have been employed are used as terms of description and not of limitation, and there is no intention in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, it being recognized that various modifications are possible within the scope of the invention.

3.3 updated LK new Sequence

SEQ ID NO: 29

10	20	30	40	50	
1234567890	1234567890	1234567890	1234567890	1234567890	
GAGCTCGCTG	CAGCCTTGAC	CTCCTGGGCT	CAAGCGCTCC	TCCCACCTCA	50
GCCTCCTGAG	TAGCTGTGAG	TATAGGTACA	TGCCACCATG	CNCAGCTAAT	100
TTTTCGATGG	TTTTTTTGTT	TGTTTTTTGT	AGTGATGAGA	TTTTCTGATG	150
TTGCTTAGGC	TGGTCTCGAA	GTCCTGAGCT	CAGGTGATCT	GGCCAGCTCA	200
GCCTCCCAAA	ATACTAGGAT	TACAGGCGTG	ANTTGGCCTG	GTCTGGTTTT	250
TCTTATATAG	GGGTCTTATC	TATATAAAGA	CTAAAGTTAA	TCTGTGCCTT	300
TGTGCGGGTG	GGCTAAGAGC	ATGATGACTT	TTATCATTCT	ATTGATTTAA	350
AGAAAACTGT	CCTTGACTTA	CCAGTGTGTA	AGTCCATGAA	AGCATAATTC	400
TGTTGAAAGC	ATATATTGTT	AATGGGTGTT	GGGAACCGTG	CACTTTCCGC	450
TGCTGTGGGA	GCATGTCCTT	GGAGGTACCT	TTCATCTGTT	TTCTCAACTC	500
CAAACATCTT	AGGACCATGG	GTTGTGACTG	GTAGGACTAT	GTATCTTGCT	550
GCTTTCAAGA	CGGAGTATAT	TTTCACGTGG	TGTCACTCTG	GCTGTCCTGT	600
TTCCCTAATA	CTGTCACTTC	ACCCTCTGCG	ATTCTGATGC	TACAAATGAT	650
AGATATCGTT	TTAGCATTTT	CTTACGGGTC	CTAGCGATTC	TATTCATTTT	700
TCTTTCAGTC	TCTTTCTCTG	ACTTGTTCAC	ATTGAACAAT	TTCCTTTTGG	750
GATAGGTTGC	TATTTCTGTT	TTCGCAGGTG	GTTTACCTGT	CTTCCCAGCC	800
AGTCACAGTG	GTCCTTGTCC	CCATGGTGGG	TCCGGGGCAA	GAGAGGGCCC	850
TGGGTTGGGG	GTGGGGTTCA	GTTGAAGATG	GGGTGAGTTT	TGAGGGGAGC	900
ACTACTTGAG	TCCCAGAGGC	ATAGGAAACA	GCAGAGGGAG	GTGGGATTCC	950
CTTATCCTCA	ATGAGGATGG	GCATGGAGGG	TTTGGGGCGT	GGCGCTGGGA	1000
ACGGCAGCCC	TCCCCAGCCC	ACAGCCGCGC	ATGCTCCCTG	NTCCCGCCTC	1050
AGTGCGCATG	TTCACTGGGC	GTCTTCTGCC	CGGCCCCTTC	GCCCACGTGA	1100
AGAACGCCAG	GGAGCTGTGA	GGCAGTGCTG	TGTGGTTCCT	GCCGTCCGGA	1150
CTCTTTTTCC	TCTACTGAGA	TTCATCTGGT	AGGTGTGCAG	GCCAGTCATC	1200
CCGGGGGCTG	AAGTGTGAGT	GAGGGTGGAG	AGGGCCTCGG	GTGGGTCAGG	1250
CGGGTCCGTT	CCTGGTCTGT	GGCCTCCGAG	GGAGAAGGGC	CACGAGGTTA	1300
CGTACCTCCT	TACCCTTCAC	AGGCTGCGAG	GCCACCGGCG	GCTTCGTGGT	1350
CGTGAAGGGG	CCTGGACGGG	GAGGAAGGTG	GGCCGTGGAG	GGGAGGCTGT	1400
CAGGGGCTCA	GGTGAAGACG	GGGTGAGTGC	TGTTGGGGGG	ATGGAAGTCC	1450
CGAGGTGCCG	GGATCCCCGA	CGACACAGGG	CAGATTCCCT	GAATGGGCCC	1500
GGCGGGGGCG	AGGCGGGCGG	TGAAGAAGGG	GCCTGGCACC	TGGGAAGGCT	1550
GCGGCCTGGC	GAGCGCCCCC	CCCAGCGGTG	TGGAGTGCGG	AGCGCCCGAG	1600
TGAGAAGCAC	TGCAAGGTCT	CACCTCCGCC	ATGGAAGGTC	CGAAAACAGT	1650
GGGAAGGAGT	GGGCGAGGCA	GTGCGGTCCA	ACCAAACTTG	TTGTGAGGGG	1700
GGGTGAATGG	CTCTAGGAAG	TGGGAGTGTG	CCCAAAGCAG	CAATCACGAG	1750
AATTGTGATT	CACTAGGGTT	TTCGTGGGGA	GTGCACTTGT	GAAACTAAAC	1800
CTCATCAGAA	ATGACCTCTG	TCTGCGGGGC	GCAGTGGCGC	TCGCCTACGT	1850

3.3 updated LK new Sequence

10	20	30	40	50	
1234567890	1234567890	1234567890	1234567890	1234567890	
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TCGAGGCTGC	AGTGAGCTGT	GATCACGCCG	CTGCACTCCA	GCCTGAGCAA	1950
CACAGCGATA	CCGCGTGTCC	AAAAGAAATT	TAGAAAAAAA	TGTCCTCTGC	2000
CTTTTGCCAC	ACGCCTTAAG	ATGATTGCTC	TGCCAGCCTG	GCCAGCAGAA	2050
GTGGCTTTGT	AGGCACTCAG	ACAGCGTACA	CACGTATGCT	TAACTCTGGG	2100
ACTTATTTTG	AGAGTATTTT	CAAAAGTAAA	ACGGCAAGTT	AACATTTATC	2150
CATGGAAGTG	ATCGAATATA	GCAGCCCTGT	GGAGCGCACG	TTCCCAATCA	2200
CGGTTGTCTG	TTTTCAGTGT	GAAATATGAG	TTGGCGAGGA	AGATCGACCT	2250
ATTATTGGCC	TAGACCAAGG	CGCTATGTAC	AGCCTCCTGA	AATGATTGGG	2300
CCTATGCGGG	TGAGTGCTTA	AACGTTAATT	CGATGTTTTC	TATTAGTAGA	2350
AATTAATTTT	TGTGATAGCG	TCGTTGCATT	AGTGTGGAAA	TGCTGATAAA	2400
GGTCTTTCCT	GCTCATAAAA	AATGAGGATG	GCATCTCATG	AAGGAAACAT	2450
TGATTCTGGA	GGATTTTTTT	TTTTCCTCTC	GTGTTCTTCA	GCTTTTGCCC	2500
ATGACTTCTT	TCTCCGGCTT	TGTTTGTTAA	TGACAGATTG	TACACATGTA	2550
TTCCAACACA	GAGTATAATA	GCCCCCAAAG	TCCTCGTGCG	TCACTTTTCT	2600
CACAGTAACC	TCCCTGTGGG	TGGAGTAACC	TTATTGGGCA	TAGAGCATAG	2650
AGTTGGAGAA	ATGTCTTTAG	GCTTAGTTAG	GACCAGAAAT	AGCTATGTAT	2700
TCTGTGTATA	TATGTAAAAT	TTTGTATCAA	TAACGAAACT	TATTTTTTAT	2750
TTGCACACCC	ACACGTATTC	CCCAGCCCGA	GCAGTTCAGT	GATGAAGTGG	2800
AACCAGCAAC	ACCTGAAGAA	GGGGAACCAG	CAACTCAACG	TCAGGATCCT	2850
GCAGCTGCTC	AGGAGGGAGA	GGATGAGGGA	GCATCTGCAG	GTCAAGGTGA	2900
GGGAAAGGGA	AGAAGAACGT	CTGCTGGTGT	GTGCGTGTGT	GTGTGTTCGT	2950
GTGTGTGTGT	GCACGTGTGT	GTGTGTTAGG	CATTGTCACA	TAGGAGGAAG	3000
AGGAGGAAAG	AAAACAATGG	AAAGAATGCC	TGAAATTGAC	TGGAAAAGCG	3050
AGGAGGCTAT	GTAGTTTGCA	GCTTAGCTTA	GGCAAATCCC	TCACTATGAT	3100
AAAAGTTCTC	GACTTTATGA	ATGAGAGAAT	GGAGGTGCCA	GGATTGTGTG	3150
TTATCCAAGA	ACCCTTGACT	GGTGAATACA	ACATTTGTAC	TGTGTTCTAA	3200
GGTTTGTGTC	TTCCTATCAT	GTATGTTGCT	GGAAAGAAGG	AAGTGATTTT	3250
GCTGAAAATG	CTTAAAACTC	AAAAGGCTTT	ACTGTAAGGT	AGCTTAGTAC	3300
TGACCCAAGA	ATAGACCCAG	TTCAGAGGAG	CAGGAGCAGC	TCCAAAAACC	3350
GAGTCGCTGA	ATGTTGGCCC	CCGTTTCCTT	TGATTGATAT	TTTTATATGG	3400
TACGTTTGAT	AAAAGCTGGA	TAAATGAGGA	TACTGCCATA	CAGGTAGCTG	3450
GTTTAGTGAT	TTTTCTCAGC	GGCCTTTAGG	AGGTGATTAA	ATCCTTTTAT	3500
GGTTAGAAAA	GCAAAAACGG	AATTATCCTG	AGATTAACGT	GAGATGGAAA	3550
TAATTTCTCC	GAGATAAAAT	GTTTTGAAAG	GAAGCATTTA	TGTAACGGAG	3600
GTCATGGATT	ATTCCAGGGA	TGCACTGTTA	AAAGTTCCTA	GAATCTGACT	3650
GACAACAATG	CCCATTAATT	GCTGTCCGCC	CACTCCCTTA	TTCTCAGTGC	3700

3.3 updated LK new Sequence

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CTAAAATATAA	CTTTATTTGA	ACAAAGTGAA	GTTTCTCTTT	ACCCCGATAG	3850
GTAATGGGTG	TCGTGACTGT	AAGATTTCCA	TAGTCCTCAA	ATCCATCCAG	3900
CTAATCAATC	CTTCAGAAAC	TGACATTGTA	ATTGTAACTG	AAATCCTACC	3950
CACGTGGTAG	ACTTCAGATT	TCTCACGTGA	CGCACACTGC	TGTTGGTACT	4000
CTAAGGCTGA	ATATAAGCAT	TATACATGTC	CTGTGGTTTA	TCCTTAGATT	4050
GTCATTTAGG	AGAAAGGTCT	AAAGCTGGGC	TGAATGCCAT	GCACTCATAG	4100
TCCCAGCTAC	TTGGGAGGCC	GAGGTGAGAG	GATTGCTTGA	GTCCTGGAGT	4150
TCAAGCCCAG	CCTGGGAAAC	ACAGTGAGAC	CTCATTGCTA	ATAAATAAAT	4200
AAATGAATAA	ATAAATAAAC	ACATAAATAA	ATTCATTAAA	TAAATAAAGT	4250
TTTCATGGTA	TAGGAAAACA	CAGATGCAAA	GTTTTTGTGC	CTAGTGGCTG	4300
GTAATGTTGC	AAACGTAACT	CCTTAGTGAA	CTGTACCACT	TAAAAATAGT	4350
TAAGATGGTA	AATTTTAGGA	TATCTGTATT	TTTTACCACA	ATTGGAAATT	4400
CCTTTCTTCC	TAAAGTTCAG	TGCAGTTATC	ATATATTCTT	TTAAATTTTT	4450
ACTGTATGTA	TCTTCAAGAC	ATAACATTCA	TAGAAAATTT	GCAAGAATAG	4500
TACAATGAAC	TCATATACTG	TTCATCTGGA	TTCACCAATG	TTAGTAGTTT	4550
CGCTTCATAG	GTTTCACATC	TCTTCCCTCC	GTCTCTTACC	GTGCTGCCCA	4600
CACACTACAC	ACACACACTC	ACACACACAT	ACGGATATAT	GTTTACTGTT	4650
ATTAATGCTG	AATTGTCTCG	ATAAAGTTTA	GGGATTATGG	TCCTTTACCC	4700
TATGTACTTG	AGGGTGTGTA	TATCGTCAGA	ACAAAGAGAA	AGTCATTTCT	4750
TGGATCCTCG	AGCTCGAGGA	TCCTGCAGCT	GCTCAGGAGG	GAGAGGATGA	4800
GGGAGCATCT	GCAGGTCAAG	GTGAGGGAAA	GGGAAGAAGA	ACGTCTGCTG	4850
GTGTGTGCGT	GTGTGTGTGT	TCGTGTGTGT	GTGTGCACGT	GTGTGTGTGT	4900
TAGGCATTGT	CACATAGGAG	GAAGAGGAGG	AAAGAAAACA	ATGGAAAGAA	4950
TGCCTGAAAT	TGACTGGAAA	AGCGAGGAGG	CTATGTAGTT	TGCAGCTTAG	5000
CTTAGGCAAA	TCCCTCACTA	TGATAAAAGT	TCTCGACTTT	ATGAATGAGA	5050
GAATGGAGGT	GCCAGGATTG	TGTGTTATCC	AAGAACCCTT	GACTGGTGAA	5100
TACAACATTT	GTACTGTGTT	CTAAGGTTTG	TGTCTTCCTA	TCATGTATGT	5150
TGCTGGAAAG	AAGGAAGTGA	TTTTGCTGAA	AATGCTTAAA	ACTCAAAAGG	5200
CTTTACTGTA	AGGTAGCTTA	GTACTGACCC	AAGAATAGAC	CCAGTTCAGA	5250
GGAGCAGGAG	CAGCTCCAAA	NACCGAGTCG	CTGAATGTTG	GCCCCCGTTT	5300
CCTTTGATTG	ATATTTTTAT	ATGGTACGTT	TGATAAAAGC	TGGATAAATG	5350
AGGATACTGC	CATACAGGTA	GCTGGTTTAG	TGATTTTTCT	CAGCGGCCTT	5400
TAGGAGGTGA	TTAAATCCTT	TTATGGTTAG	AAAAGCAAAA	ACGGAATTAT	5450
CCTGAGATTA	ACGTGAGATG	GAAATAATTT	CTCCGAGATA	AAATGTTTTG	5500
AAAGGAAGCA	TTTATGTAAC	GGAGGTCATG	GATTATTCCA	GGGATGCACT	5550

3.3 updated LK new Sequence

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CGCCCACTCC	CTTATTCTCA	GTGCGGGGGA	CAGTATATTT	TCTGTGATTC	5650
ACAAACAATG	TTATATTTGG	TGCTTTGTTG	CTTCACGGGG	TTCATTTATG	5700
GAATATTACC	TTTAGGACCT	TCGGACCTAA	ATATAACTTT	ATTTGAACAA	5750
AGTGGAAGTT	TCTCTTTACC	CCGATAGGTA	ATGGGTGTCG	TGACTGTAAG	5800
ATTTCCATAG	TCCTCAAATC	CATCCAGCTA	ATCAATCCTT	CAGACCCTGA	5850
CATTGTAATT	GTAACTGAAA	TCCTACCCAC	GTGGTAGACT	TCAGATTTCT	5900
CAGCTGACAC	ACACTGCTGT	TGGTACTCTA	GGGCTGAATA	TAAGCATTAT	5950
ACATGTCCTG	TGGTTTATCC	TTAGATTGTC	ATTTAGGAGA	AAGGTCTAAA	6000
GCTGGGCTGA	ATGCCATGCA	CTCATAGTCC	CAGCTACTTG	GGAGGCCGAG	6050
GTGAGAGGAT	TGCTTGAGTC	CTGGAGTTCA	AAGCCCAGCC	TGGGAAACAC	6100
AGTGAGACCT	CATTGCTAAT	AAATAAATAA	ATGAATAAAT	AAATAAACAC	6150
ATAAATAAAT	TCATTAAATA	AATAAAGTTT	TCATGGTATA	GGAAAACACA	6200
GATGCAAAGT	TTTTGTGCCT	AGTGGCTGGT	AATGTTGCAA	ACGTAACTCC	6250
TTAGTGAACT	GTACCACTTN	NNNNTAGTTA	AGATGGTAAA	TTTTAGGATA	6300
TCTGTATTTT	TTACCACAAT	TGGAAATTCC	TTTCTTCCTA	AAGTTCAGTG	6350
CAGTTATCAT	ATATTCTTTT	AAATTTTTAC	TGTATGTATC	TTCAAGACAT	6400
AACATTCATA	GAAAATTTGC	AAGAATAGTA	CAATGAACTC	ATATACTGTT	6450
CATCTGGATT	CACCAATGTG	GTTAGTAGCT	TTCGCTTCAT	AGGTTTCACA	6500
TCTTCTTCCC	TCCGTCTCTT	ACCGTGCTGC	CCACACACTC	ACACACACAC	6550
ACTCACACAC	ACATACGGAT	ATATGTTTAC	TGTTATTAAT	GTGAATTGTC	6600
TCGATAAAGT	TTCAGGGATT	ATGGTCCTTT	ACCCTATGTA	CTTGAGGGTG	6650
TGTATATCGT	CAGAACAAAG	AGAAAGTCAT	TTCTTGGATC	ATCACTGCAC	6700
AAAGATAAAA	ATCAGGAAAT	TTAACAATGA	GAAAATGGAG	TCATTTAATC	6750
ACAGAGTGCA	TACTCAAATT	TTGCCAGCTT	CCCCAGAAAT	TTCTTTTTTC	6800
CTTTTTTTTT	TCTTTGTTCG	AGACGGAGTC	TCTCTCTGTG	GGCCAGGTTG	6850
GAGGGCAGTA	GTGCGATCTC	GGCTCACTGC	AACCTACACC	TCCCAGGTTC	6900
TAGGGATTCT	CATGCCTCAG	CCTCCCGTGT	AGCTGGGACT	ACAGGCGCCG	6950
GCCACTGCGG	TCTTGAACTT	CTGGCCTCAC	CTGCTCTGCC	CACCTTGGCA	7000
TCCCAAAATG	TTTGGATTGC	AGGCGTGAGA	CCCCACGCCC	GGCCCAGATA	7050
ATTTTATTGA	TAGGATTTCT	TTTTCTGATC	CAGAGTCCAG	TTGAGAATCA	7100
CACCTTGCAT	GTGCTTTTCA	GGTGTTTTTA	GTTTCCTTTA	ACCTGTAATG	7150
TTTCCTTAAT	TTTTCTTGTC	ATTCACGATA	CGGACATTTT	TGGAGAGGAT	7200
AGACCAGTTG	GTTTGCAGAA	TATTCTGTAG	TTTGGGCTTT	TTCATGTATT	7250
TTAAAAGAGT	TTTCTCACTC	AGCGTTTATT	GGTGGCTACT	CATGCCATGT	7300
AAGAGTCTAA	GCGCTAGGAG	TGTAAGTGCT	GTGAGAGACG	GGATTTGAGC	7350
CTTGAGTCAT	TTAATACGAG	AAGGACAATC	AGAAGTAGAA	TAAGAGAGAA	7400

13.3 updated LK new Sequence

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GGGTNATATT	TGGAACACCT	TGTTTTCCTG	TTTCTGCTA	ACGGACTCCT	7500
GAAATAATGT	TCCTGGGATT	CTTATCAACA	CATTTATTAT	TACGTTAGCT	7550
AAAGCTTTTA	TATAATAATA	CCGAGAGCAT	GAATATCATT	TTCTTATTCA	7600
TATTTTATGT	TTTACTGCTT	AAATTGATAC	GTATTTTTTA	TTTTTAAGGG	7650
CCGAAGCCTG	AAGCTCATAG	CCAGGAACAG	GGTCACCCAC	AGACTGGGTG	7700
TGAGTGTGAA	GATGGTCCTG	ATGGGCAGGA	GATGGACCCG	CCAAATCCAG	7750
AGGAGGTGAA	AACGCCTGAA	GAAGGTAGGC	AATCCATTAG	GCATGCACAT	7800
TGTAGGGTGT	CTGTTTCCAC	AGTATCATAT	TGTAACTCTT	ACTATGTTTT	7850
TGAGACGGAG	TCTCGCTCTG	AAGACCAGGC	TGGAGTGCAG	TGGTGCCATC	7900
TCGGCTCACT	GGAAATTCTG	TCTCCAGGGT	TCAAGTGATT	CTCCTGCCTG	7950
AGCCTCTGGC	GGAGCCGGGC	TTACAGGCAT	GCTCCGCCGC	GCCCAGCTAA	8000
TTGTTGTATT	TTTAGTAGAG	ACAGGGTTTC	GTTATGTTGC	ACAGGTTGTT	8050
CCCGAACTCC	TGACCTCAGG	TGATCCACCT	GCCTCGACCA	TTGAAATTGC	8100
CGGGATTACA	GGCAGAGCCA	CCGTGCCCGA	CCCAGCATTA	TATTTTTAAT	8150
AACAGAGAGG	TAACAATACT	GCGTCTTTAG	TAACAGAGTT	CTTATATAAA	8200
GGTTATTTGA	AACGTAGTTC	AGGCCCCAGC	ACCCGGCTGA	TAGACTGTCA	8250
GATAGGGAAA	CAAAGTGAGT	CAAAGCTATG	TTGAATTAAA	AGTTTTGAGT	8300
ATAAATCCTT	AAACCAGTAG	CTCACAATTT	TCAGATGCTT	TTGTAAAGGT	8350
CTGCTTTTAA	TCAATACATA	ACACGTTTGT	AACACCCATC	ACTTGGTGTG	8400
AAAAATGCTG	AAGCACTCAT	GCGGGTTCTA	ATACCAGCTC	TTACAGCCTT	8450
GGCGAGATTC	TGAGTGAGTC	CTTTCCCTTC	TAAACCTATC	TTTGGTTCTT	8500
ATGAAAATAG	TGAGTTTAAG	TCAGAGACTT	TAAAACCATT	TTGCATTCCG	8550
TTTCTTTCAT	ACTCTGATCC	TGTTGCATAG	AATGCGTGGG	ACACAGAGAT	8600
CATCTCTTCG	CATGGTTTGT	TAATCACAAA	TCATGAAACC	CTGGCCCGAG	8650
TCATCTGAAA	ATCTCTGAAT	TGAGATTTCA	TTGTCAGTAA	GACAGTGAGC	8700
GGGCCCTCTG	CTTCATCCTA	GTTTTTCCGT	GTGGAGAGCT	GAATACGTAG	8750
TATAAGATCT	TGTGAAATTG	TGAATTCTCC	CTCTTCTTGG	TTTGTTTGTT	8800
TGTTTGCGAC	AGAGTCTCAG	TGTGTCACCC	AGGCTGGAGT	GCAGTGATGC	8850
AATTTCAGCT	CACTGCAACT	TCTGGCTCCC	AGCTAAAGCC	GTCCTCCCAC	8900
CTCAGCCTCC	CGAGTGGCTG	GAACTACATG	CACAAGCCAC	CGTGCCTGAC	8950
TACATTTTTT	TGTTTTCATT	TTTGTAGAGA	TGAGGTCTCA	CTGTGTTGCC	9000
CAGGCAGGGT	TTCTCTGGCT	TTTAATGAAC	AATTGCTTCT	TTTTTTTCCT	9050
TTTATTTATT	TATTATACTT	TAAGTTTTAG	GGTACATGTG	ACGTTGTGCA	9100
GGTTAGTTAC	ATACGTATAC	ATGTGCCATG	CTGTGCGCTG	CACCCACTAT	9150
CTCATCATCT	AGCATTAGGT	ACATCTCCCA	GTGCTATCCC	TCCCCCCTCC	9200
CCCCACCCGA	CAACAGTCCC	CAGGGTGTGA	TATTCCCCTT	CCTCTGTCCA	9250

3.3 updated LK new Sequence

10	20	30	40	50	
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TCCATGTCCC	TACAAAGGAC	ATGAACTCTT	CATTTTTTAG	GGCTGCATAG	9400
TATTCCATAG	TGTATATGTG	CCACATTTTC	TTAATCCAGT	CTATCGTTGT	9450
TGGACATTTG	GGTTGGTTCC	AAGTCTTTGC	TATCGTGAAT	AATGCCGCAA	9500
TAAACATACG	TGTGCACGTG	TCTTTATAGC	AGCATGATTT	ATAGTCCTTT	9550
GGGTATATAC	CCAGTAATGG	GATGGCTGGG	TCAAATGGTA	CAATTGCTTC	9600
TTAAATCTTT	CCCCACGGAA	ACCTTGAGTG	ACTGAAATAA	ATATCAAATG	9650
GCGAGAGACC	GTTTAGTTCG	TATCATCTGT	GGCATGTAGG	TCAGTGATGC	9700
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CTGTCAGTCT	TCATGAGCCA	CTATTTCTAA	TAAGACTGTA	GACACACATA	9800
CGATATAATC	ATCTCTAATC	ATATCAAATG	TTACATGTAA	GTTTCAGCTT	9850
TAGAGACATG	AATTGATAAG	ATTTAAAGTT	GAAAGACCAT	GACTCTAGTA	9900
CTTCCTGAGT	AATCAACTGA	AGTATGCTTT	ACACATGTGT	TTTCCAAATT	9950
GCTGACTGTT	AATTGTAAGT	GCTTGTGACT	TGAAAGGAAG	CACATGATGT	10000
TCAGGGAGGA	AATTCCTTTT	AAATTCTGCA	GGTCTACGCT	CAAAGTTTAT	10050
GCAGAGGTTC	AATTGCGTGT	AAGACACGGG	ATCACCCATA	GGGTTCTGTT	10100
TTTAGTCCAT	TTAATAAAAC	CCAAACTGTA	GTGTGCTTTG	TATGCCTTTA	10150
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GATGCAGGCT	GCTCCTATGT	TGGAAATTTG	TTCATTAAAA	TTCTCCCAAT	10300
AAAGCTTTAC	AGCCTTCTGC	AAAGAAGTCT	TGCGCATCTT	TTGTGAAGTT	10350
TATTTCTAGC	TTTTTGATGC	TGTGAAATAT	GTATCATTCT	TTGAAATCGT	10400
GTATTGTAAC	TCTCTGAGCT	GGTATGTAGA	GACATCGTTC	TTTTTTTTTT	10450
TCTTTCTTTC	TTTGTCCTCT	TTTGAGACGG	AGTCTTGCTC	TGTCGCCCAG	10500
GCTGGAGTGC	AGTGGCGCGA	TCTCTGCTCA	CTGCAACCCC	GCCTCCCGGA	10550
TTCAAGCAAT	TGTCTGCCTC	AGCCTCCCGA	GTAGCTGGGA	TTATAGGCAC	10600
CCACCAGCAC	GCCCTGGCTA	AGTTTTGTGT	TTTTACTAGA	GATGGTTTCG	10650
CATCTTGGCC	GGGGTGCTCT	TGAACTCCTG	ACCTCGTGAT	TCACCTGCCT	10700
TGGCCTCCCA	AAGTGCTGGG	ATTACAGGCA	TGCACGCCTC	CGCGCCCGGT	10750
GGAGACATAA	TTCTTACATA	TTGGTTTTCT	ATCCAGCGGC	CTTGTGAAAT	10800
ATGCTTGTGA	ATTCTAAAGT	TTACTTCTAG	GTCGTTTTCA	GTCTTCAATA	10850
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CCTCCCACAG	TGCTGGGATT	ACTGGCGTGG	GCCAGGGGCC	ACCCGTGGCG	11050
GGCCCCGTCG	TTGCCATTGT	AAAGAGTTTT	ATTTCCTTTT	CTGATTTTAT	11100

3.3 updated LK new Sequence

10	20	30	40	50	
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CTCTCCTTTT	TTTGTAGACG	GACTTTATCA	GAGTGAGTCA	TTGCATTCTG	11250
TTCCAAATTT	GCTGAGAGTA	TTCATTTGAA	TATATGTTGA	TTTTCATCAA	11300
ACAGTGCATC	TATTTCGATT	ACCACAGCGT	TTTTTCCCAT	TCATGGGTTA	11350
ATATAGTGAA	TTCGATTGAT	AAATTTGTAC	GTTTTTAGGT	TCGATTATTA	11400
AAACTTGAGA	CAGCGTCTCA	CTCTGTCACC	GAGGCTGGAG	TGCGGTGGTG	11450
TTATCAGAGC	TC				11462

We claim:

1. Isolated nucleic acid molecule which encodes a GAGE tumor rejection antigen precursor, the complementary sequence of which hybridizes to SEQ ID NO: 29 under stringent conditions.

2. The isolated nucleic acid molecule of claim 1, consisting of SEQ ID NO: 29.

3. Expression vector comprising the isolated nucleic acid molecule of claim 1, operably linked to a promoter.

4. Expression vector comprising the isolated nucleic acid molecule of claim 2, operably linked to a promoter.

5. Isolated eukaryotic cell transformed or transfected with the expression vector of claim 3.

6. Isolated eukaryotic cell transformed or transfected with the expression vector of claim 4.

7. Process for making an expression vector capable of encoding a GAGE tumor rejection antigen precursor, comprising inserting the isolated nucleic acid molecule of claim 1 into a vector which comprises a promoter, wherein said isolated nucleic acid molecule is inserted into said expression vector in operable linkage orienta-

tion to said promoter.

8. Process for making an expression vector capable of encoding a GAGE tumor rejection antigen precursor, comprising inserting the isolated nucleic acid molecule of claim 2 into a vector which comprises a promoter, wherein said isolated nucleic acid molecule is inserted into said expression vector in operable linkage orientation to said promoter.

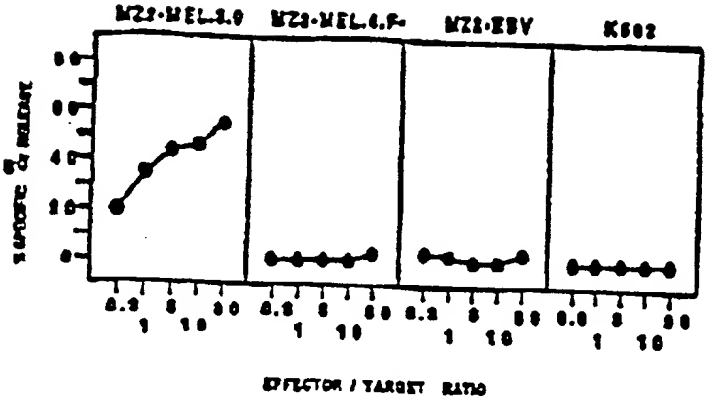

Figure 1

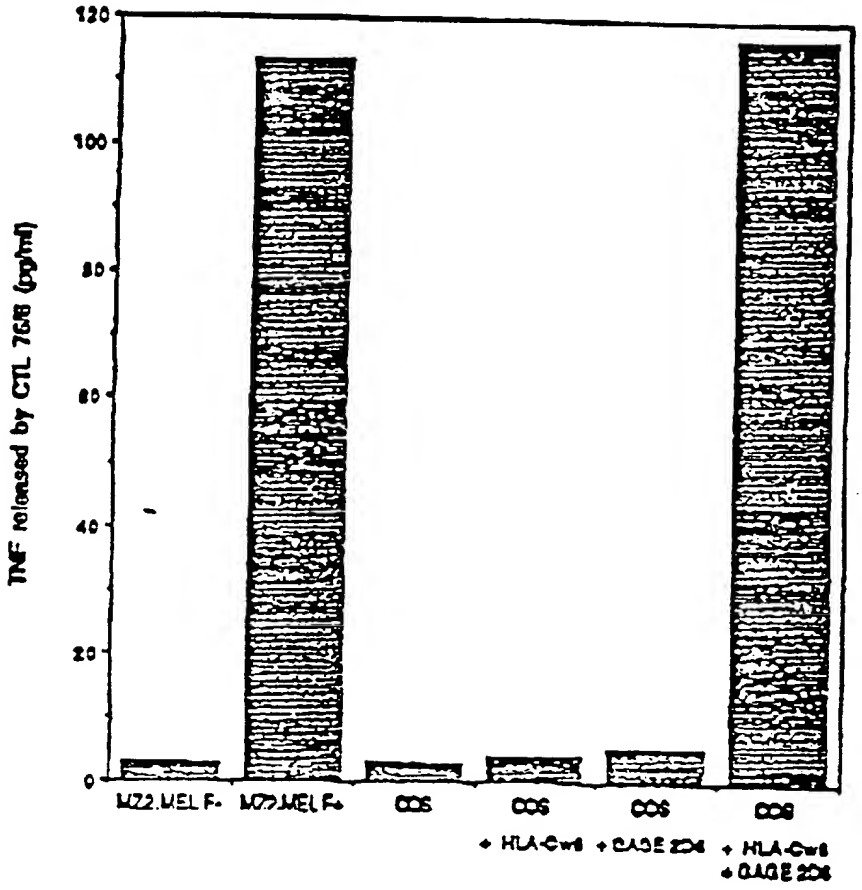
TNF released by CTL 7648 (pg/ml)
120
100
80
60
40
20
0
M22.MEL P-
M79.MEL P+
COS
COS
COS
COS
+ HLA-Cw6
+ GAGE 2D4
+ HLA-Cw6
+ GAGE 2D4

Figure 2

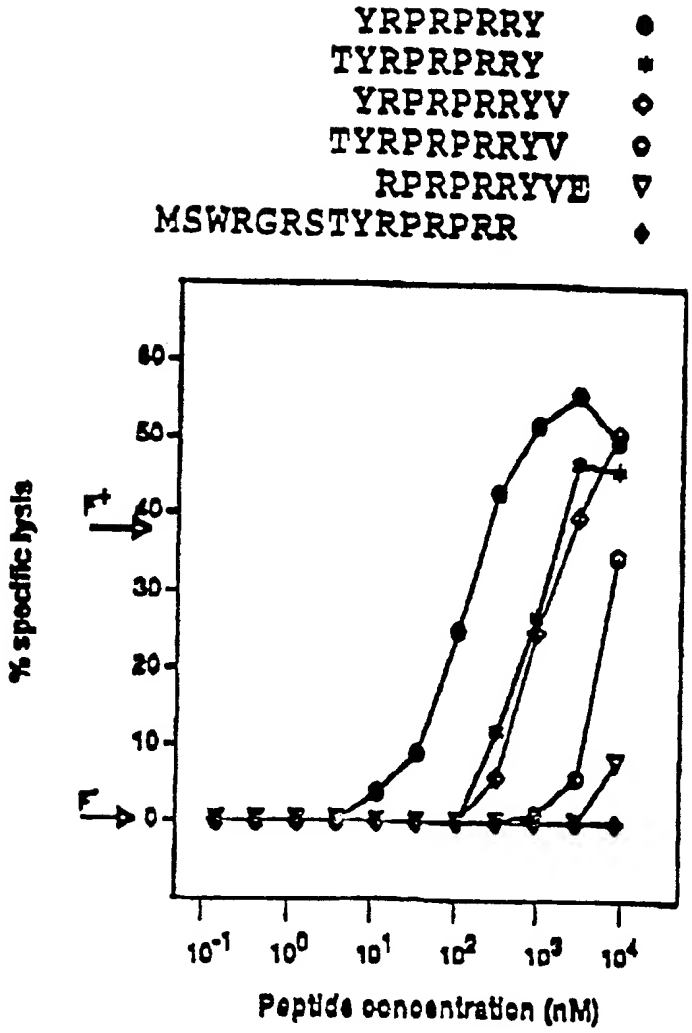
YRPRPRRY
TYRPRPRRY
YRPRPRRYV
TYRPRPRRYV
RPRPRRYVE
MSWRGRSTYRPRPRR
% specific lysis
60
50
40
30
20
10
0
F+
F-
10^{-1}
10^{0}
10^{1}
10^{2}
10^{3}
10^{4}
Peptide concentration (nM)

Figure 3

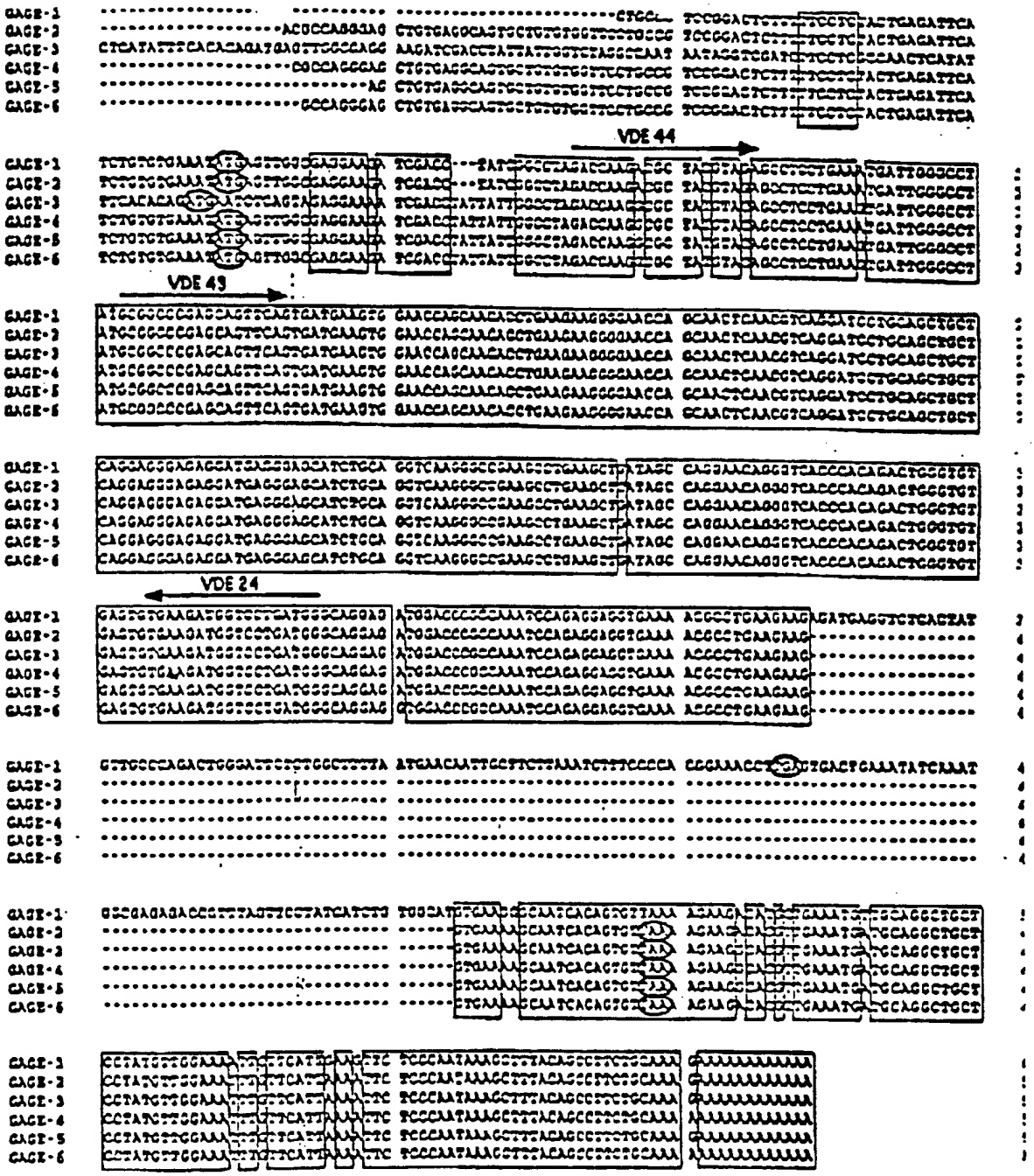

FIGURE 4

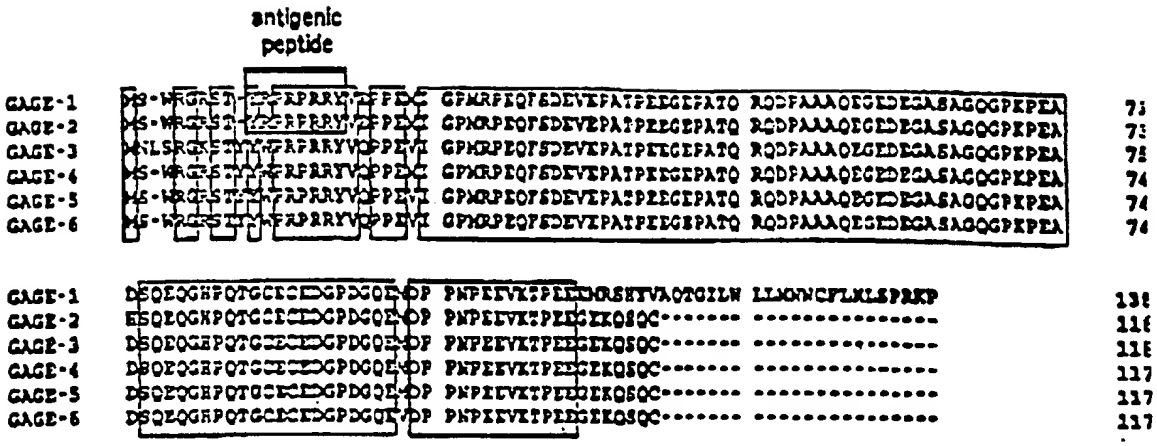

FIGURE 5

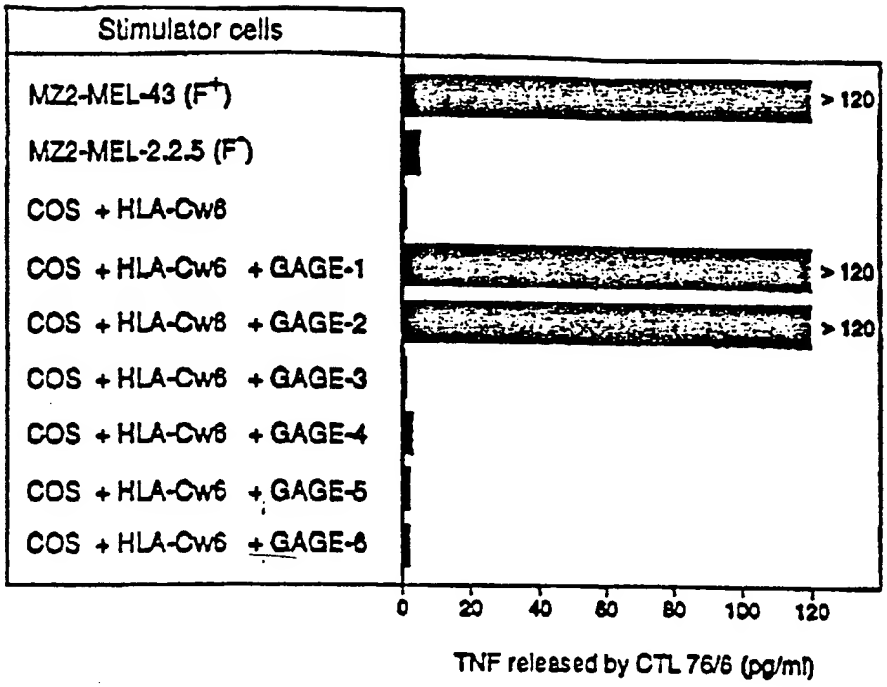
Stimulator cells
MZ2-MEL-43 (F+)
MZ2-MEL-2.2.5 (F-)
COS + HLA-Cw6
COS + HLA-Cw6 + GAGE-1
COS + HLA-Cw6 + GAGE-2
COS + HLA-Cw6 + GAGE-3
COS + HLA-Cw6 + GAGE-4
COS + HLA-Cw6 + GAGE-5
COS + HLA-Cw6 + GAGE-6
> 120
> 120
> 120
0
20
40
60
80
100
120
TNF released by CTL 76/6 (pg/ml)

FIGURE 6

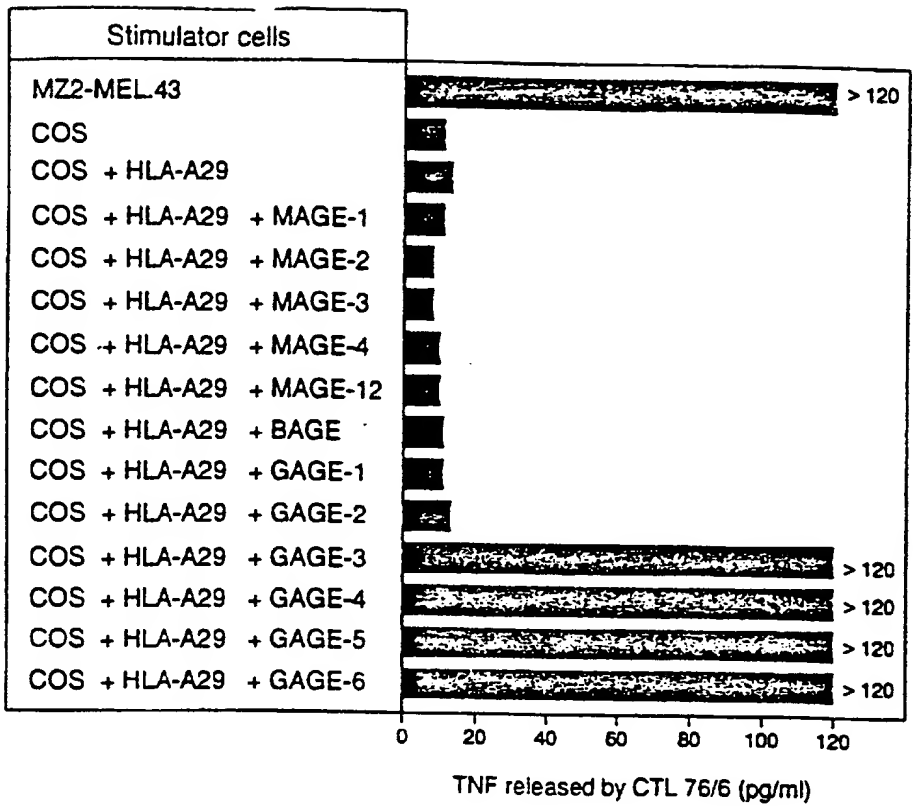

Figure 7. Stimulation of MZ2-CTL 22/23 by COS-7 cells transiently transfected with an HLA-A29 cDNA and MAGE, BAGE or GAGE cDNA. The CTL was added after 24 hours and the production of TNF was estimated 24 hours later. MZ2-MEL.43 was used as a positive control stimulator cell.

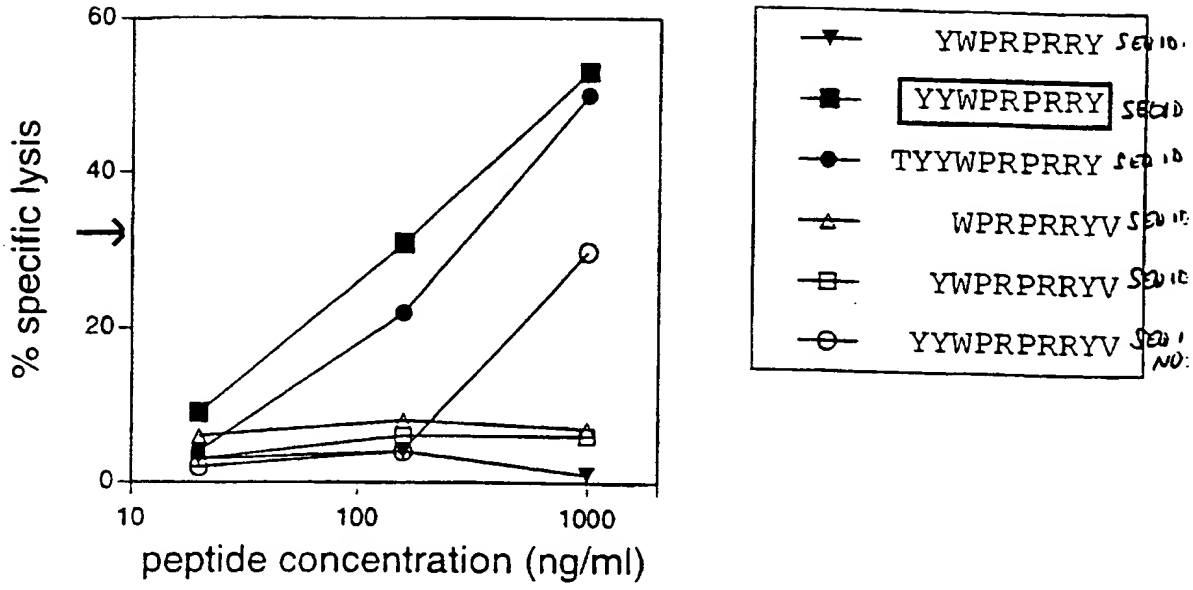

Figure 8. Lysis by MZ2-CTL 22/23 of lymphoblastoid cell line LB17-EBV incubated with GAGE-encoded peptide YYWPRPRRY. Thousand 51Cr-labelled LB17-EBV target cells were incubated in 96 well microplates in the presence of various concentrations of peptide for 15 minutes at 37°C. An equal volume containing 6000 CTL was then added. Chromium release was measured after 4 hours at 37°C. We have indicated the final concentration of peptides during the incubation of the target cells with the CTL. The arrow indicates the percentage of lysis of MZ2-MEL.43 cells.

Figure 8

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/10850

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 38/00, 45/05, C07K 7/00, 14/82; C12N 15/00
US CL :536/23.5; 435/69.3, 320.1, 325

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 536/23.5; 435/69.3, 320.1, 325

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

TUMOR REJECTION ANTIGEN FILES.

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS AND DIALOG (FILE-BIOCHEM) DATABASES: KEY WORDS: GAGE, TUMOR REJECTION ANTIGEN, DNA

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 95/03422 A1 (LUDWIG INSTITUTE FOR CANCER RESEARCH) 02 February 1995, see entire document.	1-8
Y	VAN DEN EYNDE, B. et al. Presence on a human melanoma of multiple antigens recognized by autologous CTL. Intl J. Cancer. 1989, Vol. 44, pages 634-640, see entire document.	1-8
Y	WOLFEL, T. et al. Lysis of human melanoma cells by autologous cytolytic T cell clones. J. Exp. Med. September 1989, Vol. 170, pages 797-810, see entire document.	1-8

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
27 OCTOBER 1997	05 DEC 1997
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